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9-Fluoreneacetyl-tagged, solid-phase reagent for derivatization in direct plasma injection

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ABSTRACT

We describe here a resin-based derivatization reagent, containing a 9-fluoreneacetyl tag on a controlled-pore substrate, for direct injection analysis of amphetamine in plasma. On-line, pre-column derivatization was performed by direction injection of diluted plasma sample into an sodium dodecyl sulfate-containing mobile phase. Amphetamine was trapped in the hydrophobic derivatization column and derivatized at elevated temperature by the activated solid-phase reagent. The derivatized 9-fluoreneacetyl amphetamide was separated by reversed-phase high-performance liquid chromatography with a step gradient and determined by fluorescence detection. The synthesis scheme, characterization, and optimization of the derivatization conditions for the solid-phase reagent are described. The method was evaluated by reproducibility tests and single blind spiking analysis. This solid-phase reagent combined with a surfactant containing mobile phase provided a sensitive and simple procedure for on-line derivatization in direct injection analysis of biological fluids.

INTRODUCTION

Liquid chromatography is a principal method for analysis of drugs in biological fluids [1]. Plasma, serum, urine, etc., are complex mixtures that contain a multitude of components. When analyzing for drugs, some special problems arise due to the nature of the samples. The presence of large amounts of protein in serum or plasma, the strong affinity between these proteins and drugs, the low levels and poor UV absorbance for some drugs and/or the small amount of sample available, all put high demands on effective sample preparation and chromatographic separation. Sample pre-treatment is the most laborious step and the main source affecting accuracy of the analytical procedures, especially for such biological samples. The extensive pre-treatment removal of the protein content is necessary for drug analysis in biological fluids, otherwise these proteins would denature and accumulate on the surface of conventional octadecylsilica reversed-phase packings, causing column clogging after only a few sample injections.

The sample treatment process includes extraction of the drugs or protein precipitation by an organic solvent, evaporation of the organic solvent, dissolution of the extracted drugs in mobile phase, etc. As analytes can co-precipitate with the proteins and only be partially extracted from the supernatant, inaccuracy and reduced detectability can be expected. Recently, high-performance liquid chromatography (HPLC) column packings with a hydrophilic

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outer zone and a hydrophobic inner zone were developed for direct analysis of drugs in biological fluids [1–3]. Protein precipitation is avoided by restricting the access of the larger proteins to the hydrophilic zone only.

Another way to directly analyze drugs is by using surfactants in the mobile phase [4–6]. Under reversed-phase conditions, the surfactants solubilize the denatured proteins and modify the reversed-phase column creating an external hydrophilic surface. The surfactant also helps to displace the drug from the serum proteins and increases retention by the stationary phase. Various types of surfactants can be used as mobile phase modifiers, including anionic, cationic, or non-ionic compounds. For example, the surfactant sodium dodecyl sulfate (SDS) has been used successfully, below its critical micelle concentration (CMC), for direct drug analysis under reversed-phase conditions without protein precipitation [5]. However, poor detectability and low concentrations of many analytes still prevent these direct injection methods from becoming a good tool.

By derivatization with an appropriate chromophore, separation and more importantly detection of analytes at low concentration can be drastically improved with the most commonly used detection techniques (UV, fluorescence, electrochemical detection) in HPLC [7]. Compared to derivatization in solution, solid-phase derivatization reagents have the following advantages: (i) they give more selective derivatization with fewer side products; (ii) they are non-volatile and thus have greater stability in air and during storage; and (iii) excess reaction reagents are not present with the soluble products [8]. In solid-phase derivatization, the detection-sensitive tags are immobilized on solid supports. Analytes must diffuse into the pores of the solid-phase reagent in order to react with the bonded, reactive tags. By restricting the pore size of the rigid resin, the large protein molecules are excluded from the pores. This has two effects. First, proteins cannot precipitate on the hydrophobic internal surface. Consequently, they do not block the access of analyte to the active tag. Second, the nucleophilic residues on the proteins will not deplete the solid-phase reagent of its activity. The proteins contact only the external part of the solid-phase reagent particles. Protein will stay completely in the external solution as long as the conditions causing protein denaturation and precipitation are avoided [9]. After solid-phase derivatization, low-molecular-mass analytes modified with the chromophore can be detected with high sensitivity. Detailed chemical procedures for synthesizing similar solid-phase reagents have been published [10–15].

This paper describes the preparation of a covalently bound, fluorescence (FL)-sensitive tag, 9-fluoreneacetyl (FA), on a pore size-controlled, rigid polystyrene–divinylbenzene resin (Figs. 1 and 2). Gradient elution, using SDS surfactant in the mobile phase, was applied for direct injection of plasma samples containing amphetamine. The FL-sensitive tag was covalently bonded to the resin through a nitrobenzophenol ester linkage. Derivatization conditions were tested in the on-line, pre-column mode using amphetamine. Controlled pore size of the polymeric reagent restricted the access of high-molecular-mass serum components into the pores, where the chromophore was immobilized. This restriction prevented blocking of the reagent inside the pores by protein precipitation. Low detection limits were achieved for diluted plasma without extraction. Reproducibility and accuracy of the analysis were shown by repeated injections and single blind spiked sample tests.

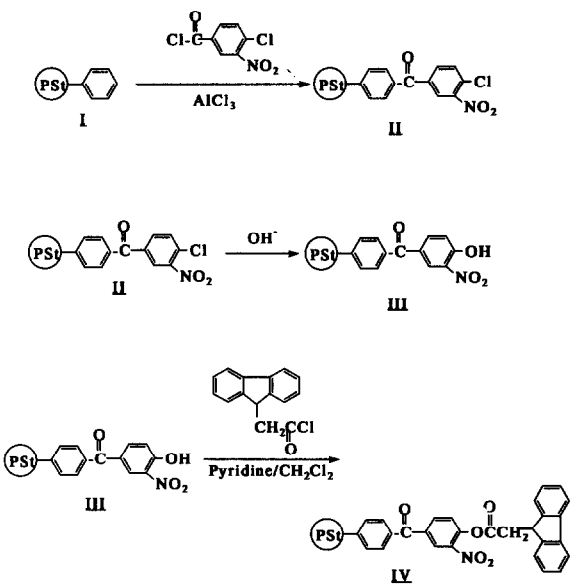

Fig. 1. Synthesis of 9-fluoreneacetyl-tagged solid-phase reagent.

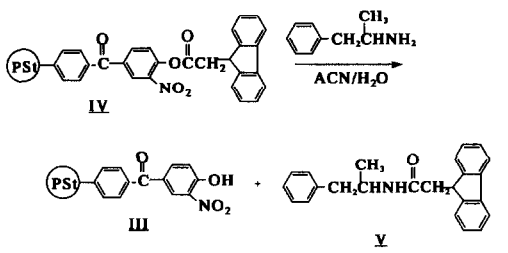

Fig. 2. Derivatization of amphetamine by FA-tagged solid-phase reagent.

EXPERIMENTAL

Reagents

9-Fluoreneacetic acid (99%), triethylamine (TEA, 98%), SDS and dichloromethane (99%) were obtained from Aldrich (Milwaukee, WI, USA). IMPAQ RG 1020 Si silica was obtained from the PQ Co. (Valley Forge, PA, USA). Omnisolv brand acetonitrile (ACN) was donated by EM Science (Gibbstown, NJ, USA). HPLC mobile phases were prepared based on volume ratio, and used after filtration through a 0.45-μm HVHP type solvent filter (Millipore, Bedford, MA, USA), and degassed under vacuum with stirring. Amphetamine sulfate was obtained from Research Biochemicals (Natick, MA, USA). Lyophilized human plasma was obtained from Sigma (St. Louis, MO, USA), reconstituted in deionized water and diluted 5-fold with 0.05 *M* NaOH before spiking. The pH of this plasma solution was 11.

Apparatus

The HPLC system consisted of a Waters 6000A solvent-delivery pump (Waters Chromatography Division, Millipore, Milford, MA, USA), two Rheodyne Model 7125 injection valves (Rainin, Woburn, MA, USA), a Waters 420 FL detector (excitation wavelength 254 nm, emission wavelength 313 nm), a LiChrospher C_{18} reversed-phase column (5 μm, 250 × 4 mm I.D., EM Science), a Supelcosil LC-ABZ column 150 × 4.6 mm I.D. (Supelco, Bellefonte, PA, USA). Data were recorded on a Linear strip-chart recorder (Linear Instruments, Reno, NV, USA), a Hewlett-Packard 3380A integrator (Hewlett-Packard, Avondale, PA, USA) or a Dynamax data system (Rainin). The UV and FL spectra were measured on an LDC Spectronic 1201 UV spectrophotometer (Milton Roy/LDC Division, Riviera Beach, FL, USA) and a Perkin-Elmer 650-105 fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT, USA), respectively.

Procedures

Synthesis

Preparation of controlled pore size polystyrene–divinylbenzene. The porous rigid resin was prepared by a templated polymerization technique of a 24:6:70 (v/v/v) mixture of divinylbenzene–ethylstyrene–styrene using trimethylsilyl-modified silica (IMPAQ RG 1020 Si, having 102 Å average pore size, 1.08 ml/g pore volume, 366 m^2/g surface area and 16–20 μm irregular particle shape) [16].

Synthesis of nitrobenzophenol intermediate. The porous rigid resin was extracted with dioxane for 8 h in a Soxhlet apparatus to remove impurities and unreacted monomers. Modification of the cross-linked polystyrene followed literature procedures, to produce nitrobenzophenol intermediate **III** (Fig. 1) [17].

Synthesis of 9-fluoreneacetyl acid chloride. To 4.0 g of 9-fluoreneacetic acid (17.7 mmol), 3.9 ml of oxalyl chloride (44.6 mmol), 30 ml of benzene (dried with anhydrous sodium sulfate) and 3 drops of triethylamine were added. The mixture was heated at 55°C for 1 h. Excess oxalyl chloride was removed by rotary evaporation. The product was dissolved in 35 ml of dichloromethane, to yield a solution of 0.12 g/ml 9-fluoreneacetyl chloride, based on total conversion of 9-fluoreneacetic acid [18].

*Preparation of nitrobenzophenol 9-fluoreneacetate **IV** (Fig. 1).* A 1.3-g amount of intermediate **III**, 4.2 ml of 9-flouoreneacetyl chloride solution (2.0 mmol), and 0.3 ml of triethylamine (2.0 mmol) in 20 ml of dichloromethane was stirred at room temperature for 1 h. The solid reagent **IV** (Fig. 1) was filtered and washed with 2 × 30 ml acetonitrile.

*Synthesis and characterization of 9-fluoreneacetyl-tagged amphetamine standard derivative **V** (Fig. 2).* A solution of 0.032 g amphetamine sulfate (0.17 mmol), 0.4 ml of TEA (1.7 mmol) and 0.18 g of 9-fluoreneacetyl chloride (0.8 mmol) in 20 ml of dichloromethane was stirred at room temperature for 2 h. The reaction mixture was evaporated to dryness under vacuum. The solid was dissolved in 40

ml of ethyl acetate and extracted with 3 × 50 ml of 0.5 *M* aqueous HCl, followed by 3 × 50 ml of 0.5 *M* aqueous NaOH. Preparative-scale reversed-phase HPLC with ACN–water (60:40, v/v) mobile phase, using a Waters Novapak 100 × 25 mm I.D. C_{18} column, a Waters WISP 712 auto-injection system and a ISCO FOXY 2150-001 fraction collector (ISCO, Lincoln, NE, USA) was used for further purification. The melting point of derivative **V** was 163.0–163.5°C. Mass spectrometry with chemical ionization was used to identify the product. Major fragments (*m/z*) were 342.3 ($M + 1^+$), 250.2, 178.1, 165.1 and 91.0. Elemental analysis results were: C% = 84.61 (84.45), H% = 6.89 (6.74) and N% = 4.08 (4.10). Numbers in parentheses represent the calculated values for $C_{24}H_{23}NO$. UV maximum was determined at 260 nm with *E* (molar absorptivity) = $2.0 \cdot 10^4$ cm^{-1} M^{-1}. Maximum excitation and emission wavelengths were 308 and 320 nm, respectively, in the FL spectrum. For fluorescence detection in HPLC, 254 nm instead of 308 nm was used as the excitation wavelength to avoid interference from the scattering of incident light.

Characterization of the tagged resin

Hydrolysis. A sample of 20 mg of solid reagent **IV** was vigorously agitated in 4.0 ml of 0.5 *M* aqueous NaOH–ACN (2:1, v/v) solution and heated at 70°C for 20 min. The released 9-fluoreneacetic acid was diluted to 80 ml after filtration and quantified by HPLC–FL. The recovery percentage of standard 9-fluoreneacetic acid was 99.95 ± 0.05% (mean ± standard deviation, $n = 3$) under hydrolysis conditions. A calibration plot of standard 9-fluoreneacetic acid was used for concentration determinations by HPLC, using 0.03% trifluoroacetic acid in ACN–water (50:50, v/v) mobile phase.

*Elemental analysis of intermediates **II** and **III** (Fig. 1).* Polymeric reagent intermediates **II** and **III** were dried to constant weight. Approximately 20 mg of each product were sent for elemental analysis (Galbraith Laboratories, Knoxville, TN, USA). The results were: intermediate **II**: C% = 85.71, H% = 7.03, N% = 1.29 and C1% = 3.47; intermediate **III**: C% = 85.99, H% = 6.67, N% = 1.27 and C1% = 0.19. Loading capacity, in mmol/g, was calculated based on: (1) decrease in chloride content from the intermediate **II** to **III**, and (2) nitrogen content in the intermediates **II** and **III**, respectively.

On-line, pre-column derivatization. A stainless-steel reaction column (35 × 2 mm I.D.) capped with a porous 2-μm frit was packed with solid-phase reagent **IV** in an ACN slurry by applying vacuum to one end. The surface was flattened by removing excess reagent particles from the column after packing, then a 2-μm frit was placed at this end of the reaction column. The packed reaction column was installed on-line in the HPLC system, between the injection valve and the analytical column (Fig. 3). A defined amount of sample was injected at room temperature. After 10 s, the second valve was rotated to trap the sample within the derivatization column. Then the derivatization column was heated in a thermostated water bath (30–85°C) for a set time. The switching valve was rotated again to wash out the reaction product and the derivatization column was cooled to room temperature. Mobile phase was continuously passed through the derivatization column, at room temperature, during the chromatographic separation. Injection and derivatization of amphetamine spiked plasma were performed in 1 m*M* SDS in ACN–water (10:90) mobile phase. The 9-FA derivative was eluted from the derivatization column by step gradient elution, from 1 m*M* SDS in ACN–water (10:90) to 1 m*M* SDS in ACN–water (55:45), for the analytical separation. Low ACN concentrations in the initial mobile phase wash avoided the precipitation of proteins and the gradient elution procedure decreased the total analysis time. Complete elution of derivatives from derivatization column has been demonstrated by using authentic 9-fluoreneacetyl amphetamide under the same gradient conditions.

RESULTS AND DISCUSSION

The purpose of this work was to develop a fluorescent reagent, covalently attached to a resin, to derivatize nucleophilic analytes during direct injection of biological fluids. Solid-phase reagents show selective derivatization of small molecules, with high yields. Controlled pore size reagent-resin and the use of SDS-containing mobile phases allow selective modification of small analytes in the presence of large amounts of protein. Here, a polystyrene–divinylbenzene resin modified with an activated FA tag was synthesized and evaluated by on-line

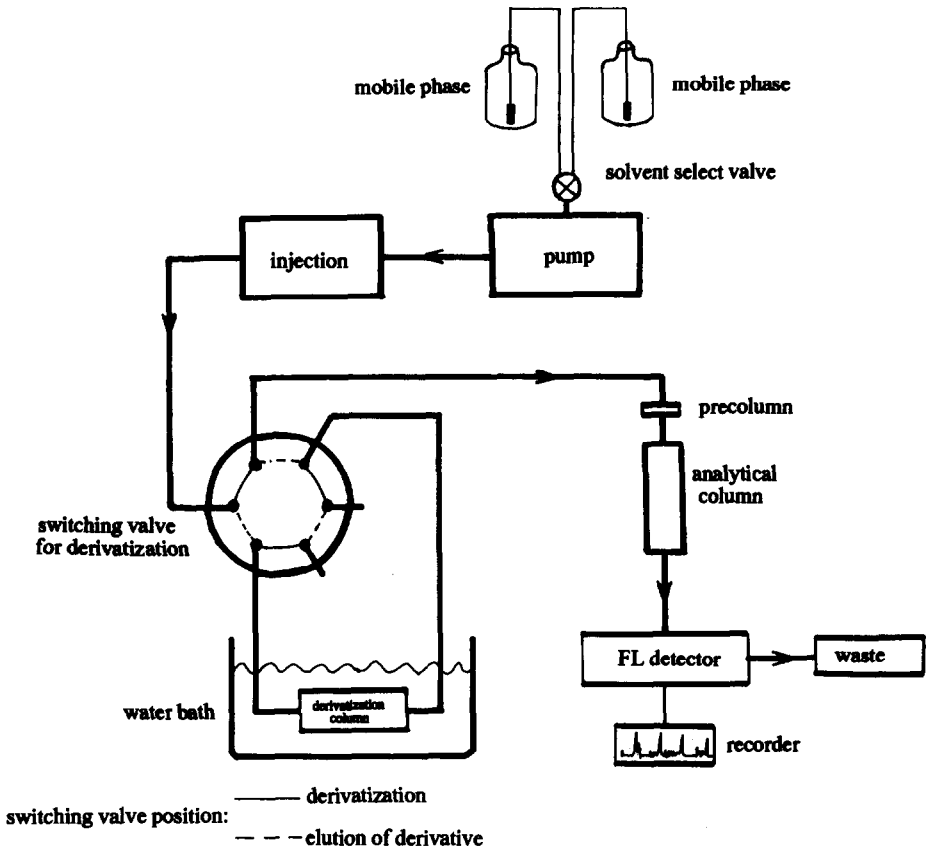

Fig. 3. Block diagram for on-line pre-column solid-phase derivatization.

derivatization of amphetamine (Figs. 1 and 2). The phenol content of the intermediate **III** was determined to be 0.92 mmol/g, based on elemental analysis results (see Experimental). Actual available loading of the FA tag on the solid-phase reagent was 0.83 ± 0.03 mmol/g (mean ± standard deviation, $n = 3$), as determined by hydrolysis of the FA tag.

Temperature and time optimization for derivatization

To obtain an efficient derivatization of amine samples, the solvent, temperature and other conditions should be optimized. Acetonitrile was found to be a good solvent for amine derivatizations [11]. A short derivatization time, combined with low derivatization temperatures, should be used to obtain a reasonably short analysis time, less decomposition of the solid-phase reagent, and higher selectivity. This combination also provides longer reagent life, lower detection limits, and fewer interferences during the chromatographic separation.

The effect of temperature was studied by using on-line, pre-column derivatization of amphetamine in ACN–water (65:35, v/v). Fig. 4 describes the conversion yield at different temperatures. At 60°C an optimum value was obtained, as shown by the turning point of the curve in Fig. 4. The time optimization curve at 60°C is shown in Fig. 5. There is no apparent increase in derivatization yield after 10 min. Based on these results, 60°C for 10 min was selected as the optimum combination for on-line derivatization of amphetamine in ACN–water (65:35, v/v) mobile phase. Under these conditions, a 70% derivatization yield was obtained for amphetamine on this resin-based reagent.

For direct injection of biological samples, espe-

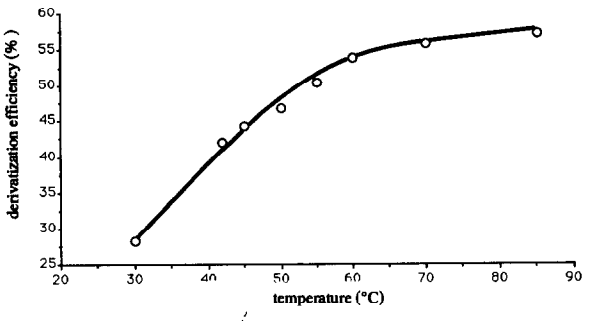

Fig. 4. Temperature optimization of on-line pre-column derivatization. Conditions: mobile phase: ACN–water (65:35, v/v); flow-rate: 1.5 ml/min; injection volume: 20 μl; sample: 10 μg/ml amphetamine in ACN–water (65:35, v/v) (pH 10); separation column: LiChrospher 250 × 4 mm I.D. ODS; FL detection: excitation wavelength: 254 nm, emission wavelength: 313 nm; derivatization column: 35 × 2 mm I.D.; derivatization time: 2 min.

cially plasma samples, onto a conventional C_{18} column, SDS containing mobile phase was used to avoid protein precipitation [5]. In this study, a 1 m*M* SDS in ACN–water (10:90, v/v) mobile phase was used for solid-phase derivatization of amphetamine spiked plasma. SDS in the mobile phase accelerated hydrolysis of the bonded reagent, particularly at elevated temperatures, resulting in more 9-fluoreneacetic acid in the front peak of the chromatogram. A lower derivatization yield (25%) was obtained in 1 m*M* SDS containing ACN–water (10:90, v/v) than in ACN–water (60:40, v/v) without SDS (70% yield), under the same derivatization conditions. To get a higher yield and reproducible derivatization in a practical derivatization time, 75°C was selected as the amphetamine derivatization temperature for the SDS containing mobile

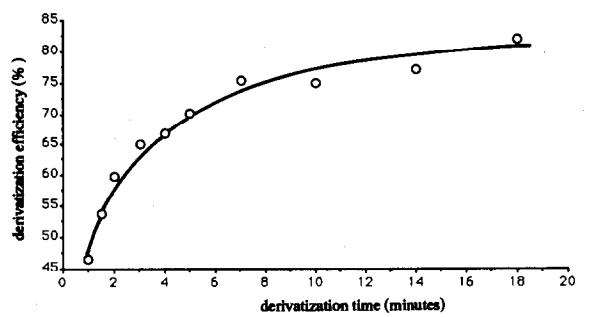

Fig. 5. Time optimization for on-line derivatization. Conditions as in Fig. 4, except derivatization at 60°C.

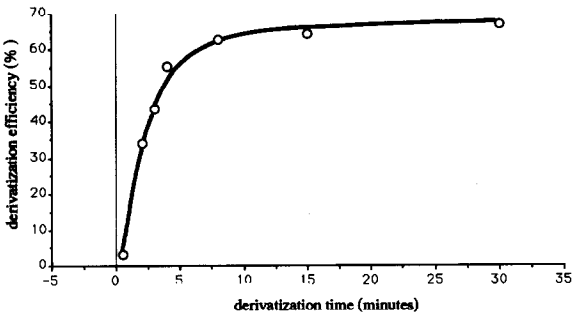

Fig. 6. Optimization of derivatization time under 1 m*M* SDS in ACN–water (10:90, v/v) mobile phase at 75°C. Sample: 20 μl of 10 μg/ml amphetamine-spiked plasma.

phase. At this temperature, the derivatization time dependency was determined for the on-line derivatization of 10 ppm amphetamine spiked plasma, as shown in Fig. 6. A reaction time of 8 min was selected as the optimum time for derivatization under these conditions.

Calibration plot for amphetamine derivatization

The calibration curve for on-line derivatization of amphetamine sample [prepared in ACN–water (65:35, v/v), pH 10] was obtained with ACN–water (65:35, v/v) mobile phase (without SDS). The relationship was linear for a 0.03–30 ppm concentration range, as shown by eqn. 1:

$$y = 22.5x \pm 0.4\ ,\ r^2 = 0.997 \qquad (1)$$

where x is the concentration of standard free amphetamine in μg/ml and y is the peak area ($\times 10^{-6}$, arbitrary units from Hewlett-Packard integrator) of the amphetamine–FA derivative from the solid-phase derivatization. Standard deviations of the slope and intercept are 0.7 and 0.4, respectively. Non-linear relationship between peak area and amphetamine concentration was observed for concentrations higher than 100 μg/ml.

The calibration curve for the on-line derivatization of amphetamine in plasma was determined from standard addition analysis of amphetamine-spiked plasma. The plasma sample was derivatized on-line in ACN–water (10:90, v/v) with 1 m*M* SDS at 75°C for 8 min. The relationship was linear for a 2–40 ppm concentration range, as shown by eqn. 2:

$$y = 3.58x \pm 0.5\ ,\ r^2 = 0.999 \qquad (2)$$

where x is the ppm concentration of amphetamine in plasma and y is peak area ($\times 10^{-6}$, arbitrary units from Dynamax data system) of amphetamine–FA derivative from solid-phase derivatization. Standard deviations of the slope and intercept are 0.06 and 0.5, respectively.

Regeneration of reagent

A given amount of the bonded FA reagent in the reaction column is used up for each sample. The tagged reagent is quickly depleted by a high concentration of amines or by derivatization at elevated temperatures, which accelerates hydrolysis. Since compound **III** is the resulting product, reactivity can be regenerated by tagging with more 9-fluoreneacetyl chloride. To demonstrate this possibility, 100 mg of the polymeric reagent was depleted off-line by derivatizing 5 samples of 1 ml 0.1% butylamine in ACN. The exhausted reagent was washed with 3 × 50 ml 0.5 *M* aqueous HCl–ACN (2:1, v/v), followed by 3 × 50 ml ACN–water (1:1, v/v) and finally dried in vacuum. The depleted reagent was regenerated off-line as described for the preparation of compound **IV**. The recovered activity was demonstrated by the percent derivatization of amphetamine. The derivatization yield was 69.2 ± 3.6% (x ± S.D., n = 3) with the fresh reagent, 5.1 ± 0.7% with the exhausted reagent, and 66.2 ± 4.6% with the regenerated reagent, respectively. Derivatization yield tests were performed on-line with 20 μl of 10 μg/ml amphetamine in ACN–water (65:35, v/v) mobile phase, at 60°C for 10 min, and expressed as x ± S.D. (n = 3). Here a fresh reagent means that the solid-phase reagent was prepared and used the same day.

Shelf life determination

Freshly prepared reagents are always needed for solution derivatizations to ensure high yields, fewer side products and reproducible results. Solid-phase reagents, on the other hand, can be stored for prolonged time periods without affecting their activity.

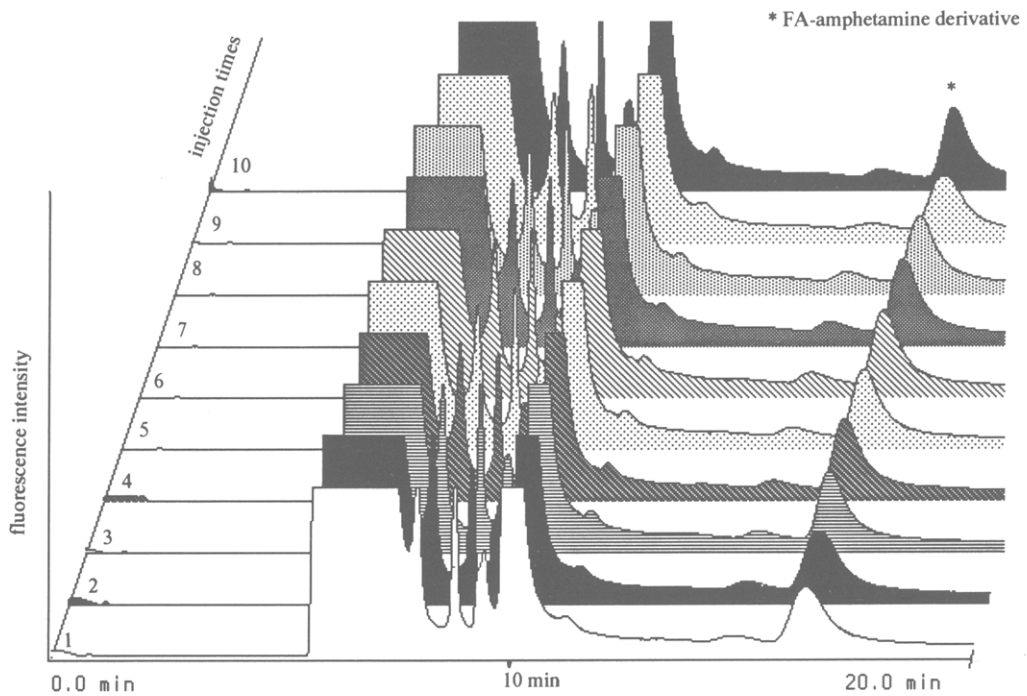

Fig. 7. Chromatograms for repeated on-line derivatizations of 10 μg/ml amphetamine-spiked plasma. Chromatographic conditions: mobile phase: step gradient from 1 m*M* SDS in ACN–water (10:90, v/v) to 1 m*M* SDS in ACN–water after derivatization; flow-rate: 1.5 ml/min; injection volume: 20 μl; separation column: Supelcosil LC-ABZ, 150 mm × 4.6 mm I.D.; derivatization column: 35 × 2 mm I.D.; derivatization temperature: 75°C; derivatization time: 8.0 min.

Activity of a freshly prepared resin tagged reagent was compared to its activity after 3 months storage in a capped glass bottle at room temperature without any special protection. Derivatization yield of a 10 μg/ml standard amphetamine solution, was 69.2 ± 3.6% (x ± S.D., n = 3) for freshly prepared reagent and 67.5 ± 2.9% for the stored reagent. These results show good storage stability of the solid-phase reagent for at least 3 months under laboratory conditions.

Reproducibility of on-line solid phase reagent derivatization of amphetamine spiked plasma

Forty 20-μl aliquots of 10 μg/ml amphetamine-spiked plasma were injected with an on-line, stop-flow, pre-column derivatization. For these 40 injections, the relative standard deviation for the derivative peak heights was 5.87%. Fig. 7 shows chromatograms of some of these runs with on-line, pre-column derivatization.

Minimum detectable amount of amphetamine in ACN–water and in plasma

The minimum detectable amount of amphetamine solutions in ACN–water and in plasma, 0.4 ng (33 ng/ml, 20-μl injection) and 4.0 ng (200 ng/ml, 20 μl injection), respectively, was attained by normalizing the signal-to-noise ratio to 3:1 with on-line solid-phase derivatizations in ACN–water and SDS–ACN–water mobile phases, respectively. Figs.

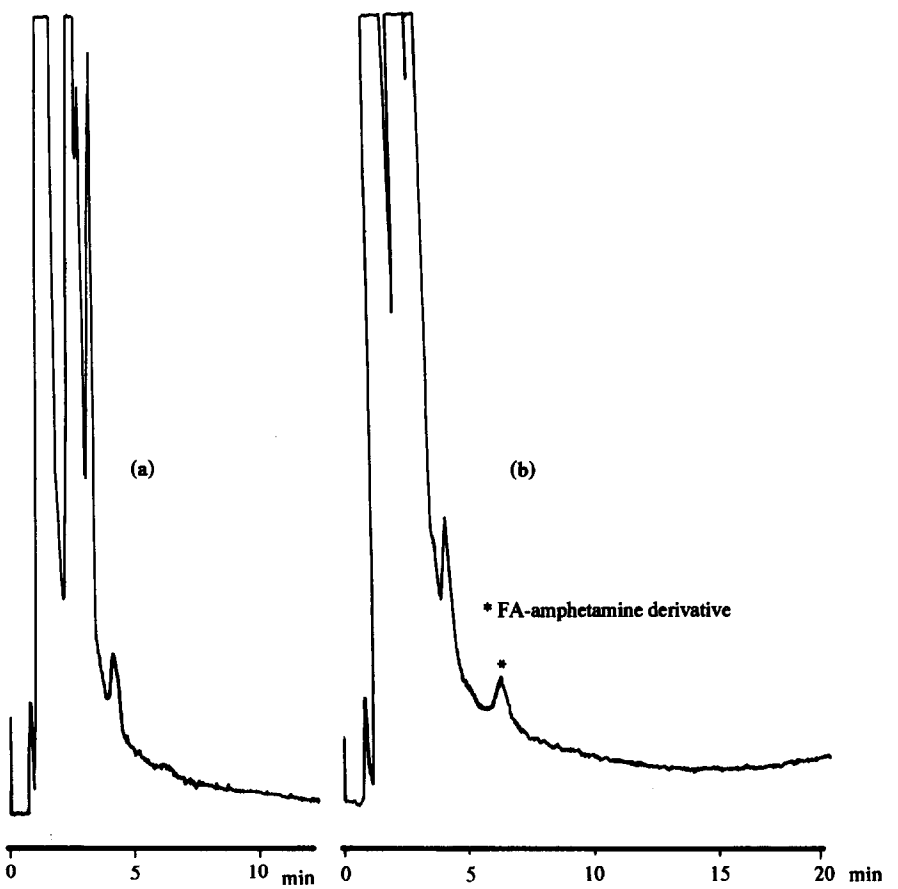

Fig. 8. Chromatogram for on-line derivatization of amphetamine in water. (a) Blank test with 20 μl ACN–water (65:35, v/v) (pH 10), (b) 20 μl of 33 ng/ml amphetamine in ACN–Water (65:35, v/v) (pH 10). Conditions as in Fig. 4, except derivatization at 60°C for 10 min.

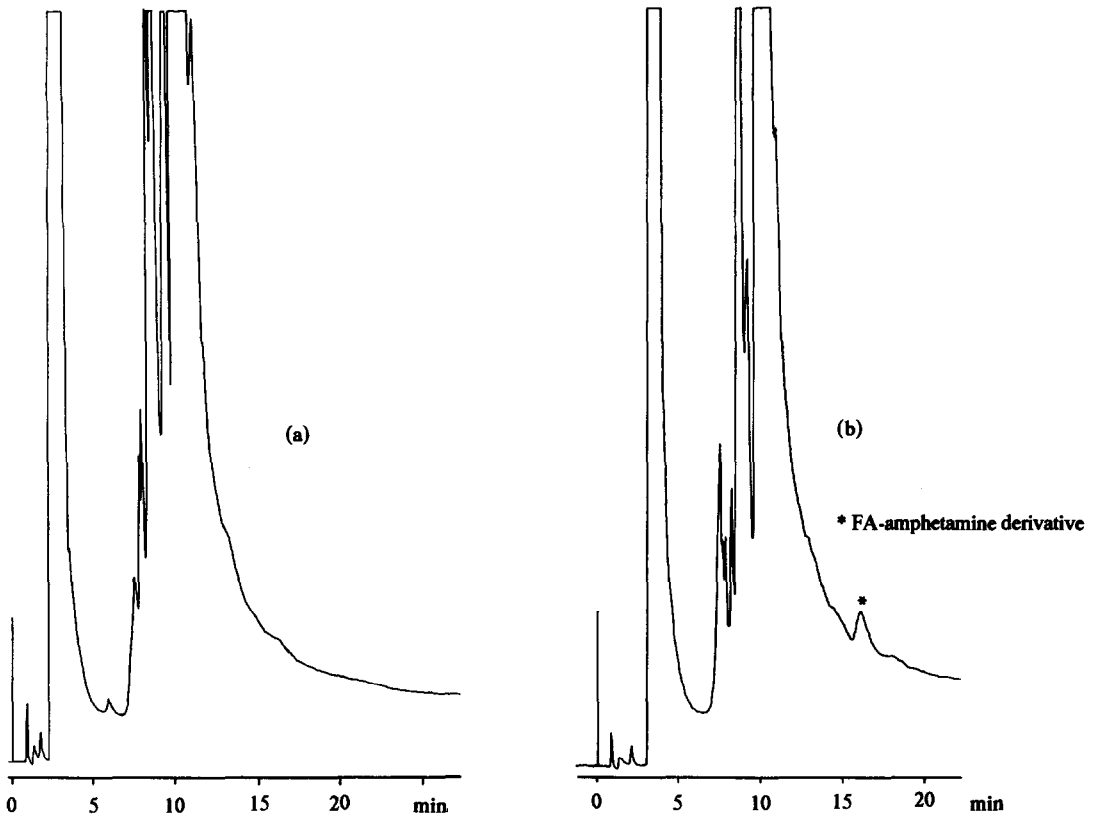

Fig. 9. Chromatogram for on-line derivatization of amphetamine in plasma. (a) Blank test with 20 μl of diluted plasma, (b) 20 μl of 0.2 μg/ml amphetamine-spiked plasma. Conditions as in Fig. 7.

TABLE I

DETERMINATION OF AMPHETAMINE SPIKED INTO HUMAN PLASMA

Derivatization conditions: 75°C, 8.0 min at 1 m*M* SDS in ACN–water (10:90, v/v); mobile phase: step gradient from 1 m*M* SDS in ACN–water (10:90, v/v), to 1 m*M* SDS in ACN–water (55:45, v/v) after derivatization; flow-rate: 1.5 ml/min; column: Supelcosil LC-ABZ 150 mm × 4 mm I.D., 5 μm; FL detection: excitation wavelength 254 nm, emission wavelength 313 nm.

Sample	Spiked (μg/ml)	Found (S.D.) (μg/ml)[a]	R.S.D. (%)[b]	RE (%)[c]
No. 1	2.25	2.36 ± 0.21	8.89	4.89
No. 2	5.87	6.29 ± 0.37	5.88	7.16
No. 3	11.52	11.80 ± 0.79	6.69	2.43

[a] Mean ± standard deviation (n = 3).
[2] Relative standard deviation = $s/x \cdot 100$.
[c] Relative error = (value found − true value)/true value · 100.

8 and 9, respectively, are the chromatograms of these low-concentration amphetamine derivatizations.

Single blind spiked amphetamine detection

To validate the use of this on-line derivatization system, three spiked plasma samples were analyzed by the standard addition method. The spiked plasma (diluted before spiking, see Experimental) was injected into a solid-phase reactor and derivatized in 1 m*M* SDS in ACN–water (10:90, v/v) at 75°C. The results are shown in Table I. The detected amphetamine concentrations were in agreement with the spiked levels.

CONCLUSIONS

We have demonstrated the synthesis and evaluation of a resin-based derivatization reagent contain-

ing 9-fluoreneacetyl tag. This reagent was used for on-line derivatization of amphetamine by the direct injection analysis of plasma. Under optimized conditions, approximately 70% derivatized amphetamine was obtained. This approach is compatible with reversed-phase HPLC separations for direct drug analysis in plasma or in other biological fluids. The overall approach was simple, accurate and reproducible. This method is fully compatible with all commercially available HPLC equipment, and in combination with an automatic sample pre-treatment system, it should have a wide range of applications [19].

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